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Article in Reviews in Medical Microbiology · March 2008
Impact Factor: 0.52 · DOI: 10.1097/MRM.0b013e32830d6046

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Laboratory diagnosis of tuberculosis: novel and nonconventional methods

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The laboratory diagnosis of tuberculosis must be accelerated and expanded not only in response to the changes in patient populations, but also in fulfillment of the need for shorter turnaround times and increased accuracy that enables conservation of limited healthcare resources. This review describes novel and nonconventional methods for the diagnosis of tuberculosis.

Laboratory testing in the field of mycobacteriology is experiencing more changes today than ever before. Determining what assays will be most useful to the clinician and tuberculosis control programs is a challenge, and acceptance of the new technology by the medical community is an even greater one. Clinicians and tuberculosis control programs must use the best available resources to determine the most appropriate care for patients, and they must interact closely with the testing laboratory. Rapid turnaround times and accurate laboratory testing must be made the highest priorities to ensure cures not only for all tuberculosis patients but most especially those with drug-resistant tuberculosis and HIV co-morbidity [1].

Recently, in order to increase the efficacy of acid-fast microscopy, a model of a computer-directed, automated microscope was constructed [2]. The automation and the time savings provided by this type of microscope can allow the reading of the required number of 100–300 viewfields and even the examination of parallel smears from the same specimen, which could well increase the sensitivity. However, this model equipment still needs further evaluation before it can be put into routine use.

As the acid-fast stain cannot differentiate between Mycobacterium tuberculosis complex (MTB) organisms and nontuberculous mycobacteria (NTM), a novel molecular biology approach using fluorescence in-situ hybridization (FISH) appears to be a promising alternative. Two peptide nucleic acid (PNA) probes targeting the 16S rRNA were developed to detect the MTB complex specifically, and NTM in general, in positive broth cultures. Potentially, this approach could find routine use on AFB-positive sputum smears [3–5]. The combined use of the two probes provides internal controls and the ability to identify MTB in mixed cultures (double infection with MTB and NTM).

The FISH assay requires a fluorescent microscope with a FITC-Texas red double filter (a standard FITC filter cannot be used due to the autofluorescence of mycobacteria); this equipment together with its maintenance can be too expensive in resource-limited countries [3]. However, in industrialized countries, the test could be an asset to the peripheral laboratory that

Direct detection

Acid-fast microscopy

Acid-fast microscopy is the fastest, easiest, and least expensive method for the rapid identification of potentially infectious tuberculosis persons. The importance of the method is further exemplified by the fact that in low-income countries, the definitive diagnosis of tuberculosis still relies solely on the presence of acid-fast bacilli (AFB) in stained smears. Although the specificity of acid-fast microscopy is excellent (all mycobacterial species are acid-fast), the sensitivity is not optimal, and the method is unable to distinguish among species within the Mycobacterium genus [1].

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DOI:10.1097/MMR.0b013e32830d6046
serves communities in which a substantial fraction of infections is due to NTMs, as no amplification equipment is needed. Although the method can readily differentiate between MTB and NTM, it is unable to identify the various NTM species. In addition, the MTB-specific probe can give a weak false-positive signal for Mycobacterium marinum, as there is only a single mismatch between the 16S rRNA sequence of this species and the sequence of the MTB-specific probe [4]. Another drawback of the method is that the NTM-specific probe does not detect the presence of the relatively commonly isolated species Mycobacterium fortuitum, Mycobacterium flavescens, and Mycobacterium xenopi [4]. These species all have more than one sequence mismatch in the capture region of the NTM probe.

**Direct nucleic acid amplification assays**

Nucleic acid amplification assays can be used directly on specimens from patients suspected of having tuberculosis. At present, there are five commercial tests offering this capability: the Amplicor PCR assay (Roche Molecular Systems, Branchburg, New Jersey, USA); the Amplified Mycobacterium tuberculosis Direct Test (AMTD; Gen-Probe Inc., San Diego, California, USA) transcription-mediated amplification method; the BDProbeTec (Becton Dickinson Diagnostic Instrument Systems, Sparks, Maryland, USA) strand displacement amplification technology; the GenoType Mycobacteria Direct test (Hain Lifescience GmbH, Nehren, Germany) transcription-mediated amplification method; and the loop-mediated isothermal amplification (LAMP) assay (Eiken Chemical Co. Ltd, Tokyo, Japan). The ligase chain reaction based LCx MTB assay (Abbott Laboratories, Abbott Park, Illinois, USA) is not approved by the FDA for use in the United States, and it was also withdrawn from the European market in 2002.

According to a recent review and meta-analysis, sensitivities of the Amplicor PCR test in respiratory specimens ranged from 83 to 96.7%, 90 to 100%, and 50 to 95.9% for all specimens, smear-positive specimens, and smear-negative specimens, respectively. When extrapulmonary specimens or mixtures of respiratory and extrapulmonary specimens were examined, the respective sensitivities ranged from 27.3 to 85%, 87.5 to 100%, and 17.2 and 70.8% for these same three categories [6]. Inhibition of the amplification reaction could be identified with the internal control of the automated Cobas Amplicor version of the test in a range from less than 1 to about 20% of the examined clinical samples [6]. The inhibition rate was shown to be significantly higher for extrapulmonary specimens. The overall specificity of the Amplicor PCR ranged from 91.3 to 100% [6]. False-positive results were obtained for specimens from patients receiving antituberculosis therapy or were related to cross-reactions with NTM [6].

Tuberculosis of the central nervous system is one of the most severe forms of human tuberculosis. The rapid and accurate laboratory diagnosis of tuberculosis meningitis is of prime importance, as the smear is often negative and cultures grow *M. tuberculosis* only after several weeks, if at all. In two recent studies, the sensitivity of the manual Amplicor PCR and of the automated Cobas Amplicor PCR, in detection of cases of definite and probable tuberculosis meningitis in patients treated for less than 10 days from whom cerebrospinal fluid (CSF) specimens had been collected, was 60% and the specificity was 100% [7,8]. The Amplicor PCR was more sensitive than the combination of smear microscopy and radiometric growth detection.

The sensitivity of AMTD in respiratory specimens varied from 85.7 to 97.8%; it was between 91.7 and 100% for smear-positive specimens. The sensitivity decreased to between 65.5 and 92.9% in smear-negative specimens [6]. For extrapulmonary specimens, the sensitivity of AMTD was between 74.3 and 100% [6]. Smear-positive extrapulmonary specimens yielded sensitivities from 88 to 100%, whereas smear-negative specimens yielded sensitivities between 63.6 and 100% [6]. Inhibition was detected in a range from less than 1 to 5% of clinical specimens. The overall AMTD test specificity ranged from 92.1 to 100% [6]. After modification of the pretreatment step for the AMTD assay, a sensitivity of 93.1% and a specificity of 97.0% were reported for tuberculosis meningitis [9]. In another AMTD study, which used a cut-off value of 11 000 relative light units, lowered from the standard 30 000, sensitivity increased from 33% to 83% and specificity remained at 100% [10]. In a more recent study examining 311 cerebrospinal fluid specimens from patients with a sound clinical suspicion, the reported sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the AMTD were 93.8, 99.3, 88.2, and 99.7%, respectively, when at least two samples were tested for each patient [11].

The sensitivity of the BDProbeTec was shown to be between 60.7 and 100% for all specimens, between 98.5 and 100% for smear-positive specimens, and between 33.3 and 85.7% for smear-negative and extrapulmonary specimens [6]. The inhibition rates of the test varied between 0.3 and 14%, showing higher percentages for extrapulmonary specimens [6]. Specificity was shown to be between 98.9 and 100%, and no cross-reaction between MTB and NTM in either respiratory or nonrespiratory specimens could be observed [6]. The BDProbeTec was also applied for the rapid confirmation of tuberculous meningitis. In a study that examined 101 prospectively collected cerebrospinal fluid samples, a sensitivity of 84.7% and a specificity of 100% could be achieved when a modified pretreatment procedure and a lower cut-off value were utilized [12].

Several studies are available on the performance of the GenoType Mycobacteria Direct test [13,14]. One study
(performed in two laboratories) revealed an overall sensitivity of 99.6 and 61.5% and a specificity of 99.6 and 100% [13]. The sensitivities for smear-positive specimens from the two laboratories were 94.6 and 85.7% and those for smear-negative specimens were 65.4 and 33.3% [13]. In another study, the reported sensitivity was 92%, the specificity was 100%, the PPV was 100%, and the NPV was 77% [14]. Sensitivity and specificity values for smear-positive and smear-negative specimens were not provided [14].

LAMP is a novel, isothermal nucleic acid amplification method that applies an autocycling strand-displacement DNA synthesis that targets six regions of the gyrB gene and 16S rDNA. The advantages of this system are its rapidity (results can be available within 35–65 min), high specificity, and lack of need for costly instruments. At present, there is limited evidence on the accuracy of LAMP for the detection of MTB [15].

These nucleic acid amplification tests can be performed in as little as 4–8 h on processed specimens, thus allowing same-day reporting of results. However, quality-control assessments indicate that these tests should be used only in laboratories that are proficient and that can afford reference reagents (including pretreatment of clinical specimens) to monitor the performance of the assays [16].

The accuracy of the in-house direct nucleic acid amplification tests varies widely. A recent meta-analysis, which examined 84 studies and used subgroup and metaregression analysis to determine sources of heterogeneity in test accuracy, found that sensitivity ranged from 9.4 to 100% and specificity ranged from 5.6 to 100% [17]. Although the use of IS6110 as the amplification target and the use of nested PCR methods both appeared to be associated with higher accuracy, the observed heterogeneity in sensitivity and specificity does not allow a clinically meaningful estimation of these tests’ reliability [17].

**Bacteriophage methods**

Bacteriophages that specifically infect and replicate in mycobacteria have also been used directly on clinical specimens to indicate the presence of viable AFB. Recently, two studies evaluated the FASTPlaqueTB (Biotec Laboratories Ltd, Ipswich, UK) assay [18,19]. In the first study, the method showed sensitivity of 87.4% and specificity of 88.2% on smear-positive specimens and sensitivity of 67.1% and specificity of 98.4% on smear-negative specimens [18]. In the second study, the overall sensitivity was found to be 75.2% and the specificity 98.7%, whereas the sensitivity for smear-negative specimens was only 54.1% [19]. It is worth noting that the gold standard in these studies was growth detection on Löwenstein-Jensen medium alone, a culture method that can give rise to false-negative results in 20–31% of specimens, unlike the more accurate combination of liquid and solid medium-based growth detection [1]. When Alcaide et al. [20] instead used an automated broth-based culture system and solid medium as the reference method, they found the sensitivity of the FASTPlaqueTB to be 58.3%, whereas the sensitivity in smear-negative specimens was only 12.1%. In a more recent study, McNerney et al. [21] described a method using mycobacteriophage D29 that showed a sensitivity of 44.1% and a specificity of 92.6%, when compared to Löwenstein-Jensen culture. However, they underlined the importance of validating the mycobacteriophage tests in settings in which infections with NTM are more common, a factor which might significantly decrease the sensitivity. In addition to these shortcomings, the conclusion of a recent meta-analysis of 13 studies on phage-based tests for the detection of *M. tuberculosis* in clinical specimens was that the performance of these tests is not superior to that of smear microscopy; the potential for contamination, and that for indeterminate results, are further unresolved issues of concern [22].

Although the phage-based systems offer a rapid and relatively low-cost diagnostic approach, these findings indicate that the current sensitivity and specificity of these tests do not support their routine diagnostic use at present.

**Growth detection**

Growth detection of mycobacteria is still indispensable, as culture is more sensitive than acid-fast microscopy, growth of those mycobacteria that are not identified by the presently available nucleic acid amplification assays is necessary for final identification, drug susceptibility tests require viable organisms, and genotyping of cultured mycobacteria is of value in a number of respects for epidemiologic purposes, to investigate the reasons behind therapeutic failure and relapse, to rule out laboratory errors, and for evolutionary analyses.

Before a culture medium is inoculated, clinical specimens from nonsterile body sites must be subjected to a pretreatment involving homogenization, decontamination, and concentration. This procedure will eradicate the more rapidly growing contaminants (other bacteria and fungi) [23]. It is important to keep in mind that the efficacy of these procedures is strongly influenced by the length of the exposure to the reagent used for decontamination, the toxicity of that reagent, the efficiency of centrifugation, and the killing effect of heat buildup during centrifugation [23]. There is evidence that even the mildest decontamination methods, such as the widely used N-acetyl-L-cysteine/NaOH method, can kill about 33% of the mycobacteria in a clinical specimen, whereas the more zealous methods can kill up to 70% [23]. In addition, if shipment of specimens to the laboratory requires longer-than-usual time (days), or for
particular patient populations (i.e., patients with cystic fibrosis, for whom *Pseudomonas aeruginosa* can overgrow the culture medium and prevent the isolation of mycobacteria), the homogenization and decontamination method to be used must be assessed carefully. The efficiency of centrifugation and the resultant yield of growth detection are also highly dependent on the quality and maintenance of the centrifuge that is used.

The guidelines of the Centers for Disease Control and Prevention (CDC) recommend that the average turn-around times for the growth detection, identification, and susceptibility testing of the MTB complex be 2–3 and 2–5 weeks, respectively, after receipt of the specimen [24,25]. The routine application of the liquid medium-based BACTEC 460TB system (Becton Dickinson Diagnostic Instrument Systems) has significantly reduced the time required to isolate MTB complex [26].

However, this procedure is still labor intensive and requires attention to the special safety and regulatory issues of radioisotopes. Therefore, several nonradiometric, fully automated or manual broth-based systems were introduced that are suitable alternatives to the semi-automated BACTEC 460TB system: the BACTEC Mycobacteria Growth Indicator Tube 960 (MGIT; Becton Dickinson Diagnostic Instrument Systems), the BACTEC 9000MB (Becton Dickinson Diagnostic Instrument Systems), the MB Redox (heipha Dr. Müller GmbH, Eppelheim, Germany), the Bio FM broth (Bio-Rad Laboratories, Hercules, California, USA), BacT/Alert 3D (bioMérieux Inc., Durham, North Carolina, USA) and the VersaTREK® Mycobacteria Detection (Trek Diagnostic Systems, Cleveland, Ohio, USA) [27–31]. Methods such as the MB Redox, Bio FM broth, and the 4ml-MGIT system still require manual processing and are best suited for laboratories that cannot afford or do not need, due to low numbers of specimens processed, automated instrumentation [28,32]. However, automation of the growth detection process is a high priority for any laboratory dealing with large specimen loads. Although the BACTEC 9000, MB/BacT, and VersaTREK culture systems provide a fully automated, walk-away growth detection process, the capacity of each of these instruments is relatively low [29–31]. Therefore, several units are required to run in parallel, an expensive set-up even for laboratories in high-income countries. The BACTEC MGIT 960 system is a high capacity, fully automated, continuous-monitoring instrument that can test up to 960 7ml-MGIT vials for the presence of mycobacteria, using a nonradiometric fluorescent technology [27,33].

Recently, another broth-based method was described to assess and follow-up the efficacy of treatment of smear-positive patients with tuberculosis [34]. This technique is based on an oxidation–reduction assay using either Alamar blue or malachite green as an indicator for the detection of bacterial growth [34]. The method is simple, cost-effective, and significantly faster than growth detection on solid medium, advantages that make it attractive for laboratories with limited resources.

In countries with higher incidence of tuberculous or other mycobacterial bacteremia, growth detection of the causative organisms from peripheral blood specimens can also be of clinical importance. It is worth noting, therefore, that with the exception of the BACTEC 9000 MB system, the novel growth–detection systems cannot be used for direct inoculation of blood. Blood samples can only be inoculated into these systems after lysis and centrifugation steps [27,30,31,35,36].

Although the broth–based systems have decreased the time to detection to 1–3 weeks, a solid medium should be used for those strains that may not grow well in liquid medium [26,28,30,31]. A further advantage for culturing mycobacteria on solid media is that growth can be quantified, colony morphology and pigmentation can be examined, and biochemical tests can be performed, if warranted. This additional information can also provide valuable clues to species identification, or it can direct the selection of other confirmatory tests such as DNA hybridization assays [37]. It is important to note that the single most important challenge of introducing growth detection in resource-limited settings is assuring that international biosafety standards are met.

**Identification**

**Nucleic acid hybridization methods**

The introduction of the AccuProbe (Gen-Probe Inc.) nucleic acid hybridization kits represented a quantum leap in the rapid identification of the MTB complex (results within 2 h, as soon as sufficient biomass is obtained following growth in culture) [38]. In rare instances, crossreaction has been documented in the AccuProbe for the MTB complex with isolates of either *Mycobacterium celatum* types 1 and 3 or *Mycobacterium terrae*, when the test is not performed precisely as indicated in the package insert [39–41]. Adherence to the proper hybridization temperature (between 60 and 61°C, rather than 60±1°C) was the most critical parameter [41]. Unfortunately, an adequately standardized protocol for the combined use of liquid media and nucleic acid probes is still lacking (the manufacturer’s instructions are for solid medium). Toward this goal, the *Manual of Clinical Microbiology* recommends that the pellet of a 1.0–1.5 ml aliquot of the broth culture should generally be used for probing, after concentration by centrifugation at 9000–10 000 × g for at least 5–7 min [38].

Because DNA/RNA probe assays do not include an amplification step, these tests are not sensitive enough to
be used directly on clinical specimens. However, DNA/RNA probes are usually capable of identifying MTB in contaminated liquid cultures (depending on the extent of the contamination), given that they have a sensitivity and specificity of nearly 100% when at least 10^3 mycobacteria are present [42,43].

**High-performance liquid chromatography**

High-performance liquid chromatography (HPLC) of mycolic acids of *Mycobacterium* spp. has proved to be a rapid and reproducible tool to identify a wide range of known or unknown mycobacterial species [44–47]. Standardized procedures and pattern standards have been published by the HPLC Users Group and the CDC (www.cdc.gov/ncidod/publications/hplc.pdf) [44,48].

A particular advantage of HPLC is the reliable distinction of *Mycobacterium bovis* BCG from *M. tuberculosis* and *M. bovis* [49]. However, HPLC cannot differentiate between *M. tuberculosis* and *M. bovis*. The most commonly used method is ultraviolet (UV) HPLC; however, the adaptation to the fluorescent HPLC can significantly increase the chromatographic sensitivity while reducing the cell mass needed and the time required for mycobacterial identification. Although not as sensitive as nucleic acid amplification assays, fluorescent HPLC is being used on a limited basis for rapid direct testing on sputum [46]. Overall, HPLC is rapid (<2 h) and the consumables are inexpensive; however, the assay requires a dedicated and highly trained technician (due to the visual interpretation of the chromatographic patterns), costly instruments and software, expertise on instrumentation maintenance, and standardized growth conditions (including the need for a large biomass in the case of UV-HPLC) [44,47].

**PCR and restriction fragment length polymorphism analysis**

The concept of differentiating among slowly growing mycobacteria by PCR and restriction fragment length polymorphism analysis (PRA) using the *hsp65* gene was developed by Plakiatys et al. [50]; ultimately, a method based on this gene was developed for use in routine clinical practice by Telenti et al. [51], Taylor et al. [52], and Devallois et al. [53]. Later, Brunello et al. [54] developed a modified PRA of the *hsp65* gene with a new algorithm describing 54 species, including 22 species that were not described previously.

This rapid test can be done on AFB isolates that grow either in liquid medium or on solid medium. Because of the amplification by PCR, the assay requires less biomass than either the AccuProbe or HPLC. A website that contains band patterns for reference for those laboratories that are using the PRA procedure is also available (http://app.chuv.ch/prasite/index.html). A drawback of PRA is misidentification due to intraspecies genetic variability (if the PRA pattern is not distinct). In order to overcome this problem, two new diagnostic algorithms were developed on the basis of the PRA of the 16S–23S DNA spacer region and on the *rpoB* gene of mycobacteria [55,56]. In addition, Kim et al. [56] targeted a novel 644-bp region of the *hsp65* for amplification; in this way, they could more readily differentiate 62 mycobacterial reference strains. This new PRA method had better resolution, and the PRA profiles of most strains were found to be distinctive enough to be interpreted visually, without an expensive computer-assisted analysis system. A multicenter study, performed in eight Latin American and Caribbean laboratories, showed that the method could be reliably implemented in resource-limited countries as well. However, improvements in gel running conditions and training in interpretation of patterns are needed in order to further improve accuracy [57].

**PCR and solid-phase reverse hybridization technology**

Recently, three commercial kit-based tests have been made available for the identification of mycobacteria. The INNO-LiPA Mycobacteria assay (Innogenetics N.V., Ghent, Belgium) targets the 16S–23S DNA spacer region for identification of mycobacteria, whereas the GenoType Mycobacterium CM (for the 14 clinically most relevant mycobacteria) and AS assays (for 16 further clinically relevant mycobacteria) (Hain Lifescience GmbH) target the 23S rDNA [58–60]. Both methods are based on the solid-phase reverse hybridization of biotinylated PCR amplicons of the target region to oligonucleotide probes arranged on a membrane strip. These systems are capable of the simultaneous detection and identification of the MTB complex and other potentially pathogenic mycobacteria from solid and liquid media. A major advantage of this methodology is the possibility of simultaneous detection of species in mixed cultures [58,59].

**DNA sequencing**

DNA sequencing of variable genomic regions offers a more rapid and accurate identification of mycobacteria than do conventional phenotypic methods. DNA sequencing methods are based on the determination of species-specific nucleotide sequences, which for identification are then compared to known sequences from in-house or commercially available databases [61]. The most routinely used and reliable method of this kind is the amplification and sequence analysis of hypervariable regions of the gene encoding the 16S rRNA [61–64]. However, a recent study demonstrated that clear-cut results with the 16S rRNA gene sequencing are not the rule, as public or commercial databases may be inaccurate or may not include all established species [65]. Therefore, alternative DNA sequencing methods and alignment algorithms have been described for the characterization of mycobacteria. These assays are based on the amplification of the *rpoB*, *gyrB*, *hsp65*, *secA1* genes, the gene encoding the 32-kDa protein, or the 16S–23S
rRNA gene spacer [66–72]. Two major advantages of \textit{rpoB} sequencing are that it can identify NTM in addition to MTB and that it can detect mutations conferring rifampin (rif) resistance in MTB in a single step [73]. Using automated sequencers, the assay can be completed and identification results can be reported within 1–3 days. However, the installation, maintenance, and running of automated DNA sequencing on a daily basis are expensive and laborious tasks.

Conventional DNA sequencing, in contrast, can be limited in its throughput, due to the length of the nucleic acid sequenced and the time needed to run optimal alignment algorithms to interpret the longer sequences. To overcome these problems, a novel method was recently developed that is based on nucleic acid sequencing during DNA synthesis (sequencing-by-synthesis). This method enables the more rapid sequencing of shorter sequences, allowing a faster turnaround time [74].

\textbf{Differentiation within the \textit{Mycobacterium tuberculosis} complex}

Each of the closely related members of the MTB complex is able to cause tuberculosis in humans. Worldwide, most cases are due to \textit{M. tuberculosis} (sensu stricto), whereas \textit{Mycobacterium africanum} and \textit{Mycobacterium canetti} are more restricted to patients living or having lived in tropical Africa [75]. Among variants with an animal host range that can affect humans as well are \textit{M. bovis} (from cattle) and \textit{Mycobacterium caprae} (from goats); in rare cases, \textit{Mycobacterium pinnipedii} (from seals) and \textit{Mycobacterium microti} (from field voles) can also cause disease in humans [76–78]. \textit{M. bovis} BCG strains, used as live vaccines or in the treatment of bladder cancer, can lead to BCG-itis in immunocompromised patients [79].

Reliable differentiation of the members of the MTB complex is required if we are to effectively address public health concerns and to make primary care decisions given these species’ differing epidemiological patterns, host geographic ranges, pathogenicities, and anti-tuberculosis drug susceptibilities. These strains’ phenotypic characteristics can be convergent or lead to indeterminate assignment if used as the only criteria for discrimination, making necessary the complementary application of genomic analysis to correctly assign an isolate to a specific member of the MTB complex. Commercially available gene probes, although they can detect the presence of MTB complex strains from specimens, are unable to differentiate among the strains, because of their remarkable genetic identity in the targeted loci, for example, the genes coding for the 16S–23S rRNA internal transcribed spacer, the 23S rRNA, or the 16S rRNA. At present, three categories of genomic targets are suitable for such discrimination: the single nucleotide sequence polymorphisms (SNPs), the regions of difference (RDs), and the spacers between direct repeats in the direct repeat (DR) region [80].

DNA sequencing analysis identified discriminatory SNPs in the \textit{oxyR}, \textit{pncA}, \textit{gyrB}, and \textit{hsp65} genes that enabled the distinction of certain members of the MTB complex [80,81]. Detection of SNPs from cultures can be performed by rapid tests such as allele-specific PCR and/or digestion of PCR products, followed by restriction fragment length polymorphism (PCR–RFLP) analysis. Although all of these rapid methods relying on SNP targets are easy to integrate into the routine of laboratories already using amplification procedures, they are unable on their own to differentiate among all members of the MTB complex. Allele-specific PCR tests that detect a unique SNP at position 285 in \textit{oxyR} gene rapidly differentiate \textit{M. caprae}, \textit{M. bovis}, and \textit{M. bovis} BCG from the other members of the MTB complex [82]. In the same way, PCR assays detecting the allele polymorphism at position 169 in the \textit{pncA} gene differentiate \textit{M. bovis} and \textit{M. bovis} BCG from the other members. The combined analysis of the two genomic targets identifies \textit{M. caprae}. However, these tests still do not differentiate among \textit{M. tuberculosis}, \textit{M. africanum}, \textit{M. canetti}, \textit{M. microti}, or \textit{M. pinnipedii}. \textit{M. canetti} can be rapidly differentiated from the other MTB complex members by PCR–RFLP of the \textit{hsp65} gene [83]. A PCR–RFLP assay targeting five discriminatory regions in the \textit{gyrB} gene was able to correctly identify \textit{M. africanum}, \textit{M. microti}, and \textit{M. caprae} using a unique genetic marker [84]. \textit{M. tuberculosis} and \textit{M. canetti} display identical \textit{gyrB} DNA sequences and are indistinguishable by this test, as are \textit{M. bovis} and \textit{M. bovis} BCG. However, because the isolation of \textit{M. canetti} is rare, this may not pose a major problem when the analysis is performed on strains isolated from non-African populations. The putative inability of \textit{gyrB} DNA gene and other genetic markers to differentiate \textit{M. tuberculosis} from \textit{M. africanum} subtype II has been recently resolved given the taxonomical classification of the latter as a phenotypically atypical \textit{M. tuberculosis} [85–88].

The DR region is another genomic target suitable for the discrimination of the members of the MTB complex. This region contains a variable number of direct repeats and a variety of DNA spacers between the direct repeats. Because most of the members of the MTB complex have signature features in the DR region, they can be easily differentiated by spoligotyping, on the basis of the presence or absence of specific spacers [85,89–91]. Spoligotyping is a simple, inexpensive, and robust PCR-based method that in its original form involved the amplification of the whole DR region, followed by the hybridization of the amplified DNA to a set of 43 spacers covalently linked to a membrane [92]. The method has the clear advantage of simultaneously discriminating the
members of the complex and also to differentiating the clinical isolates in one assay. Spoligotyping has the additional advantage that it can recognize genotypic families among MTB complex isolates; notable are the Beijing strains, which are highly prevalent in Asia and may be responsible for severe outbreaks of multidrug-resistant tuberculosis [MDR; defined as resistance to at least isoniazid (INH), and RIF]. For instance, the highly drug-resistant W strain, first identified in New York, is a member of the Beijing family [93]. However, some MTB complex strains show ambiguous spoligo patterns, making necessary further analysis for discrimination. Although spoligotyping has been used successfully to detect and type M. tuberculosis in clinical specimens, the patterns were not reproducible [94]. Reproducibility from cultures is 94% [95]. As the method requires multistep hybridization procedures, it may not be within the scope of a clinical diagnostic laboratory. In addition, commercially produced membranes for spoligotyping are most efficiently used for assaying 40 isolates per run. For increased flexibility and cost-effectiveness, the original membrane-based spoligotyping has been transferred to the Luminex Multianalyte Profiling System (Austin, Texas, USA), which uses microspheres that contain two fluorochromes and that are interrogated by two lasers [96]. The Luminex system can be used for 1–96 isolates without increasing the labor time or cost per isolate. This system may be an attractive one for laboratories that frequently require a rapid turnaround time for only a few isolates per run. As yet, however, only one report on the system’s effectiveness is available [96].

Comparative genomic studies recently revealed RD in the members of the MTB complex; these regions represent sequential losses of genetic material in the course of the evolution of the MTB complex [97–99]. These highly conserved RDs are either common to various members of the MTB complex or specific to individual members. Two PCR-based approaches that detect the presence or absence of RDs have been evaluated for differentiation of the members of the MTB complex. Each RD was independently amplified with specific primers, and the amplification products were then visualized by agarose gel electrophoresis. Because these genomic deletion assays are rapid and simple to perform, they can be incorporated into the laboratory routine by many clinical mycobacteriology laboratories. The first approach, which considered six RDs (RD1, RD3, RD5, RD9, RD10, and RD11), was able to accurately differentiate M. tuberculosis and M. africanum (RD10 positives), and M. bovis and M. bovis BCG, in a large collection of MTB complex clinical isolates [88]. This approach has the advantage that it can resolve most identifications with an initial screening of only RD1, RD9, and RD10. A further advantage is that these three regions are tested by multiprimer PCR, which results in each case in an amplification product with a size indicating whether the RD is present or not present and avoids false-negative amplifications. However, that assay could not differentiate M. africanum RD10 negatives from M. microti, and it did not evaluate the identification of M. caprae and M. canetti. The second genomic deletion approach was based on five other RDs (Rv1510, Rv1970, Rv3877/78, Rv3120, and IS1561); it correctly differentiated seven members of the MTB complex, M. tuberculosis, M. canetti, M. africanum, M. caprae, M. bovis, and M. bovis BCG [86]. However, it requires multiple PCR tests, and false-negative amplifications can lead to misidentification. PCR-based RD deletion analysis of RD4, RD9, and RD10 has also shown an excellent ability to distinguish M. tuberculosis and M. africanum strains with ambiguous spoligotypes [100].

The GenoType MTBC (Hain Lifescience GmbH) test is the only commercial kit available for the differentiation of clinical MTB complex isolates. This DNA strip assay is based on a combination of gyrB gene SNPs and the RD1 deletion of M. bovis BCG [101–103]. Specific oligonucleotides targeting these polymorphisms are immobilized on membrane strips. Amplicons derived from a multiplex PCR react with these probes during hybridization producing specific patterns. The system correctly differentiates M. tuberculosis, M. africanum, M. microti, M. caprae, M. bovis, and M. bovis BCG. However, M. tuberculosis and M. canetti are indistinguishable from one another in this assay. The assay is fast, with minimal technical requirements, and can easily be incorporated in a manual or automated manner into the routine of laboratories already using amplification procedures.

### Novel and molecular susceptibility testing methods

In recent years, several new and rapid methods have been developed to meet the need for immediate susceptibility testing results. However, some of these assays are still at the developmental stage.

### Inhibition of growth

Recently, the fluorescent MGIT system was evaluated in a multicenter study, to determine susceptibility of isolates to the four first-line drugs, in a comparison to the radiometric assay [104]. No statistically significant difference was found between the results of the two methods. The advantages of the MGIT system are its elimination of use of the radioisotope 15C, and its ready availability in both manual and automated formats [105].

The fully automated VersaTREK system, which works by detecting pressure changes within the headspace above the broth culture medium, has also been reported to be capable of generating rapid and reliable INH, RIF, ethambutol (EMB) and pyrazinamid (PZA) test results.
In the similarly fully automated and continuously monitoring MB/BacT system, a colorimetric CO₂ detection sensor indicates mycobacterial growth. Data from a recent multicenter study using modified critical concentrations for all five first-line antituberculosis drugs showed that the system is a rapid and reliable method for INH, RIF, and PZA susceptibility testing of MTB complex; however, the lower sensitivity for EMB and streptomycin indicates that further improvement is needed [107].

It is worth noting that a recent review on susceptibility testing performed with the automated nonradioactive systems discerned a major drawback in many published studies and evaluations, namely, the lack of testing of an adequate number of drug-resistant isolates. The review recommends the inclusion in any study of at least 30% drug-resistant isolates [108].

Detection of metabolic changes
Viability assays based on the use of indicator dyes have been used to determine the drug susceptibility status of mycobacteria. In 1958, Pital et al. [109] used resazurin as an oxidation–reduction indicator, enabling them to report susceptibility test results for streptomycin and INH within 5–8 days. Yajko et al. [110] revisited this concept and compared the Alamar blue dye assay to the agar proportion method, finding a 97% overall agreement. In a recent study, resazurin was used to detect MDR tuberculosis; 7-day MIC results for INH and RIF agreed with results obtained with the proportion method [111]. Another dye, 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), is reduced by dehydrogenases in living cells to produce insoluble purple MTT formazan crystals, which after solubilization can be measured spectrophotometrically. This assay has been adapted for use in determining viability of MTB cells following interaction with RIF [112,113]. In a more recent study, the well known characteristic of MTB to reduce nitrate to nitrite was used in a microplate assay to determine the MICs for INH, RIF, and EMB within 8 days; results were compared with those from the Alamar blue assay and the conventional agar proportion method [114]. It has been suggested that these simple, fairly rapid, and inexpensive screening methods are appropriate for use in countries where funds for more expensive assays are not available.

Semi-quantitative analyses measuring products of metabolism have also been developed. Flow cytometry, comparing the DNA content and number of mycobacterial cells after 1–3 days in the presence or absence of drug, has been used to determine susceptibility of tubercle bacilli to antimycobacterial agents [115,116]. In addition, relatively rapid assessments of mycobacterial viability in the presence of drugs have been performed using bioluminescence to measure mycobacterial ATP levels [117,118].

Bacteriophage methods
The luciferase reporter mycobacteriophage technique has been shown to be capable of distinguishing drug-resistant from drug-susceptible strains of MTB in a 48-h assay [119]. The percentage of residual light activity is proportional to the percentage of the mycobacterial population that is viable and able to support replication of the infecting bacteriophage and, consequently, expression of the phage-encoded luciferase gene. Bacterial cells that are capable of producing light in the presence of a specific drug are determined to be resistant to that drug [119–121]. One modification of this assay utilizes a Polaroid film box, called the Bronx Box, as a rapid and simple means of photographic detection of light from lytic phage-infected MTB [121].

The PhaB (biologically amplified phage) assay is based on the ability of viable MTB bacilli to protect infecting mycobacteriophage from chemical inactivation and thus to support the replication of the infecting phage [122]. Progeny mycobacteriophage released following lysis of mycobacterial cells are detected by infection and subsequent lysis of the rapidly growing sensor strain, Mycobacterium smegmatis. In a comparative study on 133 MTB complex clinical isolates, the commercial product FASTPlaque TB-RIF (Biotec Laboratories Ltd) was reported to provide RIF susceptibility results comparable to those from the BACTEC 460TB within 2 days, without the need for specialized equipment [123]. In a more recent study, an in-house version of the method using mycobacteriophage D29 was evaluated on 102 MTB complex isolates against the proportion method using Löwenstein–Jensen. The sensitivity of this in-house assay was 97.8% and the specificity was 100% [124].

However, results of a recent meta-analysis on RIF susceptibility testing of clinical isolates with bacteriophage methods, analyzing data from 19 studies, revealed that although these tests have a relatively high sensitivity, the specificity is more variable [125]. The latter carries the potential of false overdiagnosis of RIF-resistant tuberculosis. In addition, sensitivity and specificity of the tests when performed directly on sputum specimens vary widely, in the two studies published thus far [126,127]. Therefore, such tests currently are not considered to be accurate enough for routine diagnostic application.

Evaluation of RNA levels
Detection of tRNA by the AccuProbe DNA probes can be used as an early index of the metabolic activity of MTB because the photometric light units can be correlated with the numbers of colony forming units per milliliter [128]. High photometric light unit readings after incubation in the presence of the drug indicate resistance.

Bacterial mRNA is typically short lived, with a half-life of only a few minutes. Recently, a novel reverse-transcriptase strand-displacement amplification system was
developed for the detection of MTB mRNA directly in patient sputum samples [129]. The loss of detectable mRNA corresponded to a rapid drop in the numbers of viable organisms present in each sputum sample over the first 4 days of treatment, allowing an early, rapidly, and effective patient monitoring. However, as a caveat to this approach, Hu et al. [130] found that both microaerophilic stationary-phase MTB treated with a high dose of RIF in vitro and PZA-induced persistent bacteria in mice still have transcriptional activity, even though they are nonculturable (similarly to dormant MTB in individuals with latent tuberculosis infection).

**Molecular susceptibility testing methods**

Genetic studies have determined that in MTB, resistance to antitubercular drugs is the consequence of spontaneous mutations in genes encoding either the target of the drug or enzymes involved in drug activation. Resistance-associated random chromosomal mutations have been described for all first-line drugs [131,132]. However, no single genetic alteration has yet been found that results in the poly-resistant or MDR phenotype. Rather, poly-resistant or MDR develops through sequential acquisition of mutations at multiple loci. Unfortunately, the multiple locations of the mutations for each drug, and the fact that resistance to a particular drug can involve a mutation in more than one gene (i.e., INH and the katG, inhA, kasA genes) or several distinct loci of the same gene (i.e., RIF and the rpoB N-terminal and 81-bp core region mutations), have made the molecular detection of drug resistance a challenging task.

For example, INH resistance-associated mutations have been found in the mycobacterial catalase gene (katG), and in genes encoding enzymes that participate in synthesis of the cell wall mycolic acids (i.e., inhA, kasA) [132–134]. However, approximately 15–25% of INH-resistant isolates do not contain mutations in any of these regions, indicating that other sites must also be involved in resistance to this drug. Likewise, only about 70% of EMB-resistant clinical isolates of MTB contain mutations in embB, the drug target involved in biosynthesis of the cell wall component, arabinan [132–134]. RIF resistance is more easily assessed using molecular methods, as more than 96% of the RIF-resistant isolates of MTB contain mutations in a well-defined, 81-bp (27-codon) central region of the gene encoding the β-subunit of RNA polymerase (rpoB) [132–134]. However, mutations associated with RIF resistance can also occur in other regions of the rpoB gene, albeit less frequently (i.e., V146F mutation in the N-terminal region) [135]. Similarly for PZA-resistance, up to 97% of all PZA-resistant clinical isolates tested carry a mutation in the putative promoter region or the structural gene (pncA) that encodes for PZase, the enzyme responsible for activation of the PZA pro-drug [132–134]. Therefore, selection of the method used to detect mutations associated with drug resistance in MTB can be challenging. Because DNA sequencing of the amplified product not only detects but also identifies the specific mutation, this method has the potential to serve as the gold standard. DNA sequencing also differentiates between mutations that result in an amino acid change and those that are silent. Most of the non-DNA sequence-based mutation detection methods are unable to make this distinction. Recently, an rpoB-based PCR and DNA sequencing method showed a sensitivity of 92.3% when used on smear-positive specimens and a sensitivity of 100% when used on Cobas Amplicor PCR or IS6110 PCR positive specimens directly [136]. However, use of DNA sequencing for the precise detection of mutations is labor intensive and requires a high level of expertise as well as expensive equipment. Therefore, to shorten the turnaround time entailed by the individual and consecutive resistance-associated gene sequencing and to decrease the associated costs, Yang et al. [137] applied DNA sequencing in conjunction with a single multiplex allele-specific PCR assay, for the detection of INH, RIF, and EMB resistance simultaneously.

In most mutation-detection methods that do not use DNA sequencing, validation of assays has usually begun by testing for RIF resistance, because of the clinical significance of resistance to this drug and its association with MDR TB, and also because nearly 100% resistance is due to mutations in the 81-bp region of a single target gene [132]. The assays are based on DNA and RNA research techniques, and some amplification method is used, most often PCR. The PCR product can be tested for resistance-associated mutations by digestion with restriction endonucleases, followed by gel analysis to detect fragment size differences (RFPL); direct gel analysis to detect single strand conformation polymorphisms (SSCP) caused by DNA sequence changes that result in structural changes; or temperature-mediated heteroduplex analysis by denaturing HPLC [138–141]. Another successful approach is to detect mutations using hybridization, either with allele-specific nucleic acid probes or with PNA probes in ELISA-based methods [142]. The PCR-based reverse-hybridization line probe assay (LiPA; Inno-LiPA Rif. TB Test, Innogenetics N.V., Ghent, Belgium) has been reported to be an easy-to-use test for the rapid detection of RIF resistance, especially for clinical laboratories that lack the capacity to perform DNA sequencing [73,143]. The LiPA kit consists of 10 oligonucleotide probes. One is specific for the MTB, whereas five partially overlapping wild-type probes span the 509–534 region of the rpoB gene. Four other probes are specific for amplicons carrying the most common rpoB mutations: D516V, H526Y, H526D, and S531L. Therefore, this single test is suitable for both the reliable identification of the MTB complex and the rapid and accurate detection of RIF resistance [73,143]. The test
also showed adequate sensitivity when it was used directly on smear-positive specimens [143–145]. Another commercially available DNA strip assay is the GenoType MTBDR (Hain Lifescience GmbH), which is capable of the simultaneous detection of RIF resistance-associated mutations in the 81-bp core region of rpoB (similarly to the LiPA) and the presence of INH resistance-associated mutations in codon 315 of katG [146]. The sensitivity of this test was found to be 99% for rpoB and 88.4% for codon 315 of katG-associated mutations, with a specificity of 100% for both genes. This assay was also reliably applied directly on smear-positive specimens in both industrialized and resource limited settings [147]. The GenoType MTBDRplus, besides testing for rpoB and katG mutations, also includes reaction zones for inhA mutations. This assay was evaluated in a busy routine diagnostic laboratory in Cape Town, South Africa on AFB smear-positive sputum specimens and was found to have sensitivity of 98.9% for detection of RIF resistance, and 94.2% sensitivity for INH resistance, and 98.8% for detection of MDR TB, while specificity was 99.4%, 99.7%, and 100% respectively [148].

These findings led the World Health Organization to endorse molecular line probe assays for rapid screening of patients at risk of MDR tuberculosis. The use of these tests on AFB smear-positive specimens may save resources by allowing the performance of culture and drug-susceptibility testing only when the molecular test result predicts the presence of MDR tuberculosis [149].

Recently, Brown et al. [150] reported a similar but in-house developed macroarray that detects mutations in the RIF resistance-determining region of the rpoB and in the INH resistance-determining locus 315 of the katG and locus 15 of the mabA-inhA in parallel, following a multiplex PCR. In this test, the capture probes are applied to a nylon membrane rather than to strips. The method showed a high sensitivity and specificity, is significantly less expensive than the commercial strip-based techniques, and does not require specialized instrumentation [150]. Therefore, it could be useful in areas with a high incidence of MDR tuberculosis.

The most rapid new method, real-time PCR, uses hybridization with fluorescence-labeled probes or molecular beacons during amplification; this hybridization can be followed by fluorescence-probe melting profiles to detect resistance-associated mutations [138–140,151–153]. The real-time PCR method was successfully applied for the detection and identification of MTB and its resistance to INH and RIF; both in clinical isolates and directly in smear-positive specimens [154,155].

Another promising development is the use of oligonucleotide, or DNA, microarrays. The microarray serves as a platform to which are anchored a large collection of DNA capture probes. These DNA probes are used to hybridize complementary sequences of DNA or RNA in the sample being analyzed. For hybridization to DNA chips, the DNA or RNA target in the sample must be amplified and labeled with a fluorescent dye for detection with a scanning fluorometer [156–158]. Because microarrays have a capacity of hundreds of thousands of probes, a single chip can be used to identify the species of Mycobacterium and to detect mutations in all known target genes for antimycobacterial drugs.

The potential of the DNA chip technology was also demonstrated by two recent studies [159,160]. The use of the GeneChip DNA probe array (Affymetrix, Santa Clara, California, USA) was based on information derived from two sequence databases. The first database provided species identifications (16S rDNA sequence patterns) and the second provided information on detection of RIF resistance in MTB (rpoB alleles). The same hybridization conditions could be used for the two genes and the platform that was described can be expanded to enable the simultaneous analysis of other genes as well. Epidemiological markers could also be added to the array for tracing transmission links between strains. In two more recent studies, microarray methods were successfully applied for the rapid detection of INH and RIF resistance following a multiplex PCR and for the rapid detection of INH, RIF, and streptomycin resistance following an allele-specific PCR [161,162]. With the potential to perform one-step testing of clinical specimens for mycobacterial identification, drug susceptibility, and genotyping, the DNA chip platform represents a promising advance for the clinical mycobacteriology laboratory [163].

An alternative approach is to assess whether mutations in a gene have interfered with enzymatic activity of a protein required for drug activation. Suzuki et al. [164] used PCR to amplify pncA and then synthesized PZase using an in vitro transcription–translation coupled system. The measured PZase activity was then compared to that of the wild-type gene. This system has the advantage of eliminating the need to grow a large biomass to test for PZase activity, and it also eliminates the need for DNA sequencing.

### Methods for DNA fingerprinting of the Mycobacterium tuberculosis complex

A large number of DNA-fingerprinting methods for the typing of MTB complex isolates have been developed in recent years. Most of them focus on repetitive DNA elements such as insertion sequences IS6110 and IS1081 or short repetitive DNA sequences such as the DRs [92], the polymorphic GC-rich tandem repeats [165], the exact tandem repeats (ETRs) [166], and the mycobacterial
interspersed repetitive units-variable number of tandem repeats (MIRU-VNTRs) [167]. Because each of the typing methods has significant drawbacks, only a few have been adopted for widespread use. In addition, the interpretation of molecular typing results in any setting requires thorough evaluations with the locally circulating MTB population in mind.

In the most widely used fingerprinting method, IS6110 RFLP, both the number of copies and the location of the IS6110 insertion element generate variations in the RFLP pattern. The stability of IS6110-RFLP patterns is high because of the low transposition efficiency of IS6110, an issue particularly important in the study of chronic diseases such as tuberculosis. The transposition rate is stimulated by exposure to microaerobic environments, a typical condition of dormant bacilli. This trait explains differences of one or two IS6110 copies in the patterns of strains isolated from a single patient with relapse or from different patients involved in an outbreak [168]. IS6110-RFLP typing is highly discriminatory for most M. tuberculosis strains, but it is both expensive and labor intensive, and inter-laboratory comparability of fingerprints is difficult, despite an international standardization consensus [169]. In addition, because the time lag between the isolation of the MTB complex and the acquisition of typing results is often too long to meet clinical and public health needs, more rapid and economical PCR-based methods focusing on IS6110 have been introduced in the last few years. The most reproducible (100%) one, which also has a differentiation level close to that of IS6110-RFLP, is mixed-linker PCR [170]. The key feature of this method is its ability to amplify multiple restriction fragments containing IS6110 sequences and variable sequences adjacent to the restriction site. As expected, it is not accurate for strains that have few IS6110 copies. A similar, less complex method (ligation-mediated PCR) has also been found to be highly discriminatory for clinical M. tuberculosis strains [171]. However, ligation-mediated PCR showed only 81% reproducibility in duplicate DNA samples analyzed in separated series, thus restricting its use to comparisons carried out in the same experiment [172].

The most widely used PCR-based typing methods targeting loci other than IS6110 are spoligotyping (see above) and MIRU-VNTR typing. These methods rely on PCR amplification of short repetitive sequences. In addition to being faster and applicable to crude DNA extracts, they have the advantage of providing typing data in a portable numerical format that enables easy direct comparison of results from different laboratories.

Analysis by spoligotyping of 43 spacers in the DR region has a differentiating power lower than that of IS6110-RFLP when high copy-number strains are being analyzed, but it is superior to the latter method for the evaluation of low copy-number strains [95]. Spoligotyping is therefore useful either as a prescreening method, to reduce the number of isolates to be typed by complementary typing methods [173], or as a secondary typing method for M. tuberculosis isolates with low copy-number of IS6110 [174]. Spoligotyping has been recommended as well for typing of M. bovis isolates, which characteristically contain low numbers of IS6110 copies [175]. A second-generation spoligotyping membrane, introducing 51 new spacers, was recently developed [90]. This new membrane improved the differentiation of M. bovis, M. caprae, and M. tuberculosis belonging to the Beijing genotype, whereas other M. tuberculosis strains were not further differentiated. Differentiation of M. africanum can also be improved through the use of 25 of these new spacers [85].

VNTR typing relies on PCR amplification using primers specific for flanking regions of the VNTR and on determination of the amplicon sizes, which reflect the number of amplified VNTR copies. Initial VNTR typing systems for MTB complex strains made use of very limited sets of loci [166,176] and proved not to be sufficiently discriminatory [95]. More extensive sets of VNTR loci have been described subsequently, including a system based on 12 loci that contain VNTRs of original genetic elements named MIRUs [177,178]. MIRUs are located mainly in intergenic regions interspersed in the MTB complex genome. A VNTR-MIRU-based high-speed genotyping system was developed, combining analysis of four multiplex PCRIs for the 12 loci on a fluorescence-based DNA analyzer with computerized automation [167]. The VNTR-MIRU typing can be performed as well in a manual manner more suitable for the routine of most clinical laboratories. This method exhibited about the same discriminatory power as IS6110-RFLP in most of the studies for strains with several IS6110 copies [167,179,180] and it was more efficient than IS6110-RFLP for low copy-number M. tuberculosis strains [181]. However, this typing method seems to be less discriminatory than IS6110-RFLP for strains of recently expanded M. tuberculosis families in high-prevalence countries [182]. Because of its feasibility, high reproducibility, and discriminatory power, MIRU-VNTR typing is progressively replacing IS6110-RFLP, the current gold-standard typing method.

New methods that analyze the whole genome of the MTB complex isolates have been recently developed. The amplified fragment length polymorphism (AFLP) method uses PCR to selectively amplify defined subsets of DNA restriction fragments from across the whole genome. In the fluorescent form of the method (FAFLP or fluorescent-AFLP), one of the PCR primers is fluorophore-labeled, making the amplified fragments visible to an automated DNA sequencer. In a first evaluation, FAFLP analysis was found to yield results that were precise, reproducible, and congruent with those of IS6110-RFLP typing [183]. However, the method
subsequently appeared to be nonreproducible in a blinded evaluation with a collection of international MTB complex strains; none of the duplicate DNA samples scored concordant results [172].

More promising results for detecting polymorphisms have been obtained with DNA arrays. High-density DNA microarrays, such as the Affymetrix–GeneChip system, have recently been applied to identify genomic deletions in \textit{M. tuberculosis} clinical isolates [184] and polymorphisms in the DR region similarly to spoligotyping [163]. The first results suggest that the system is suitable for epidemiological studies, although large-scale evaluations for the reproducibility of the method will still be required. Because they require an expensive technology, access to DNA microarrays remains restricted to laboratories in high-income countries.

All of the PCR-based methods have the potential to be applied directly to smear-positive specimens, providing the opportunity for real-time typing. However, the technical pitfalls should not be underestimated, especially those due to the presence of PCR inhibitors and the use of multiplex PCR. Moreover, large-scale evaluations must be carried out before these methods can attain routine application.

**Detection of false-positive cultures**

The diagnosis of tuberculosis initiates a complex series of expensive medical interventions among which are respiratory isolation, multidrug therapy, and investigation of close contacts. Because the occurrence of false-positive cultures is infrequently recognized by laboratory and clinical personal, tuberculosis control activities are sometimes inappropriately applied to patients who do not actually have tuberculosis [185]. False-positive cultures were identified in 13 of 14 studies that evaluated more than 100 patients; the median rate of false-positive cultures was 3.1% [186]. When suspected, false-positive cultures can be confirmed by use of DNA fingerprinting on all isolates manipulated within 7 days [187]. Causes of false-positive cultures include laboratory cross-contamination (contamination with an AFB-positive specimen or with control H37Rv strains) and contamination in the clinical setting of devices like bronchoscopes. A contaminated instrument can also infect other patients, so bronchoscopic contamination can be a cause of both false-positive cultures and actual tuberculosis transmission [188,189].

**Detection of mixed infections**

Although most specimens collected from patients with tuberculosis show the presence of a single strain, both HIV-negative and HIV-positive individuals can be infected with more than one strain during a given episode of tuberculosis. Mixed cultures can result from laboratory cross-contaminations as well. An average of 17% of the new tuberculosis cases in high-incidence settings can be multiple infections [190,191]; these can seriously confuse subsequent interpretation of drug-susceptibility testing results, as well as interpretation of epidemiological connections among patients [192]. MIRU-VNTR typing appears to be a useful rapid tool for resolution of such problems [193].

**Risk factors for recently acquired tuberculosis**

Molecular typing of MTB complex isolates has the capability to determine whether a particular patient is part of a cluster. It is widely accepted that such a finding is indicating recent infection with a strain in current circulation. Based on this interpretation, several molecular epidemiological studies have been conducted in low-incidence countries, either in individual large cities such as New York, San Francisco, Paris, and Hamburg [194–197], or on a nation-wide scale (the Netherlands) [198,199]. These studies made it clear that the relative contribution of recently acquired disease to the tuberculosis burden in many settings is far greater than had been thought. Risk factors for acquisition of tuberculosis following recent infection have therefore been identified in order to better address public health interventions. Risk factors varied among the studies, because such factors depend in part on the targeted population. They generally include drug abuse, male gender, young age, alcoholism, homelessness, urban residence, being foreign-born, and having pulmonary tuberculosis. However, molecular typing is not an appropriate approach to the identification of risk factors either in rural environments or in high-incidence countries with a stable population, because in these areas, the presence of particular ancestral, well conserved strains with stable molecular patterns can confuse the epidemiological interpretation [192].

**Exogenous reinfection versus endogenous reactivation**

Patients with recurrent tuberculosis can either be infected with a given strain and relapse due to its reactivation or, in contrast, they can be re-infected by a different strain after curative treatment. The correct distinction between these alternatives is critical for accurate estimation of the success rates of tuberculosis control programs. DNA fingerprinting has demonstrated on one hand that endogenous reactivation of \textit{M. tuberculosis} can occur after more than 30 years of latent infection [200], and on the other hand that prior tuberculosis disease does not provide all patients with adequate protective immunity against exogenous reinfection, which occurs more commonly than previously believed. Re-infection is an important cause of relapse in prisons [201] and in areas with high [202], moderate [203], or low incidence of tuberculosis [204].

**Failure of therapy and drug resistance**

The mechanisms by which drug resistance evolved among drug-susceptible \textit{M. tuberculosis} strains during antituberculosis treatment have been investigated in
several studies [192,201,205,206]. Unchanged IS6110 DNA fingerprints in sequentially susceptible and resistant isolates indicated development of resistance (acquired resistance). Shifted fingerprints demonstrated re-infection with a new, drug-resistant M. tuberculosis strain. Re-infection and super-infection with such strains can explain such phenomena as delayed responses to antituberculosis treatment and inconsistent drug-susceptibility patterns. Exogenous re-infection can occur either during therapy for the original infection or after therapy has been completed [207].

**Transmission links**

The availability of molecular-typing methods has facilitated the tracing of outbreaks of tuberculosis in situations when traditional contact tracing would not be able to identify the source of infection. Molecular typing has also allowed identification of previously unrecognized outbreaks and the confirmation of suspected transmission in the community, as well as in specific settings such as hospitals, prisons, schools, shelters, nursing homes, or bars [93,189,194,197].

**Follow-up of characterized strains**

RFLP-IS6110 and spoligotyping results obtained in large-scale national and international studies can be now adequately compared, following the recent adoption of standardized international protocols. Several international electronic databases containing genotyping data from thousands of MTB complex strains are under construction. Data from spoligotyping databases have already underlined the importance of international transmission of tuberculosis and have contributed to the determination of the occurrence and biogeographic distribution of emerging genotypes on a world–wide scale, for instance, the Beijing genotype [208,209]. Supported by the European Union and in association with the European Register of MDR tuberculosis cases, the European database of RFLP-IS6110 patterns of MDR isolates allows the detection and study of possible cross-border transmission of MDR tuberculosis [210].

**Interferon-γ assays for the immunodiagnosis of latent tuberculosis infection and tuberculosis disease**

One-third of the world’s population is latent tuberculosis infected (LTBI), and in 10% of these individuals, this infection will lead to active disease during their lifetimes. However, if the infected individuals are immunocompromised (e.g., HIV infected) 8–10% of them will develop tuberculosis disease within a year [211]. Therefore, it is imperative to accurately diagnose and treat patients with LTBI and also to predict who among the infected will develop the disease. The currently used tuberculin skin test (TST), which measures a delayed-type hypersensitivity response to the purified protein derivate (PPD; a crude mixture of antigens from the members of MTB complex and also NTMs) has a low sensitivity (e.g., in patients with either immunosuppression or very advanced disease) and specificity (e.g., in BCG-vaccinated individuals or in NTM-exposed populations), despite its very low cost [212]. A further disadvantage of TST is that the administration and reading of the test require expertise and results can be variable.

The recent introduction of interferon-γ (IFN-γ) tests provided an alternative for the diagnosis of LTBI. Currently, two commercial assays are available, the Quantiferon–TB assay (Cellestis Ltd., Carnegie, Victoria, Australia) and the T SPOT-TB assay (Oxford Immunotec, Oxford, UK) [213,214]. These tests measure the IFN-γ release of T cells after stimulation by *M. tuberculosis* specific antigens via an enzyme-linked immunosorbert (ELISA) or enzyme-linked immunospot (ELISPOT) assay. The first-generation Quantiferon-TB is a whole-blood test that measures IFN-γ release to purified protein derivative (PPD) with ELISA; this test was approved by the US Food and Drug Administration (FDA) [214]. The Quantiferon-TB Gold test is the enhanced form of the assay, which uses the *M. tuberculosis* specific ESAT6 and CFP10 antigens instead of PPD. This test also received FDA approval [215]. An even newer version of the test is the Quantiferon-TB Gold InTube assay, which entails simpler sample preparation, is further enhanced by the addition of TB7.7 (p4) antigen, and also received FDA approval. The T SPOT-TB assay, which requires the separation of peripheral blood mononuclear cells, detects IFN-γ release to ESAT6 and CFP10, with ELISPOT received FDA approval as well. A major drawback of any of these methods is that the incubation with the antigens must be initiated within 8–12 h of blood collection.

According to a recent systematic review of the performance of these commercially available assays, as well as a significant number of in-house assays, IFN-γ tests that use a cocktail of *M. tuberculosis* specific antigens may offer several advantages over the conventional TST [216]. These advantages are higher sensitivity and specificity, better correlation with MTB complex exposure, lower cross-reactivity with *M. bovis* BCG and NTM, and the potential to identify individuals with LTBI who are at elevated risk to develop active disease [216]. However, in the absence of a true gold standard, the reliable determination of sensitivity and specificity is very difficult. In addition, there is still inadequate evidence on the accuracy of the IFN-γ tests for HIV positive and other immunocompromised patients, for children, for extrapulmonary tuberculosis patients, for MDR tuberculosis patients, for patients with NTM infection; also uncertain is their accuracy in the monitoring of patient response to treatment [216,217]. Their cost benefit is not well proven either, although two recent studies indicate that IFN-γ tests may be cost-effective and positively affect the control of the disease [218,219]. Therefore, the potential use of these assays in
the clinical routine awaits further confirmatory studies. It is also likely that IFN-γ tests are more appropriately performed in the routine clinical laboratory than in the mycobacteriology laboratory.

Goal

The Institute of Medicine report ‘Ending neglect: the elimination of tuberculosis in the United States’ [220] reviews the lessons learned from the neglect of tuberculosis between the late 1960s and the earlier 1990s and reaffirms a commitment to the goal of eliminating tuberculosis in the United States, which is defined as a case rate of less than one case per million population per year. The report states, ‘to meet this goal, aggressive and decisive actions beyond what is now in effect will be required’, and offers a quote from Goethe: ‘Knowing is not enough; we must apply; Willing is not enough, we must do’ [220].

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